AD	

Award Number: DAMD17-99-1-9172

TITLE: Functional Analysis of TGF-a/b Regulated Protein (TGFRP)

in Breast Cancer Angiogenesis

PRINCIPAL INVESTIGATOR: Ryan M. Anderson

CONTRACTING ORGANIZATION: Duke University Medical Center

Durham, North Carolina 27710

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;

Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Burden Perspective Perspective (7/04-0189). Washington, DC 20503

Management and Budget, Paperwork Reduction Proje		[A Bara == == == ==			
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE		REPORT TYPE AND DATES COVERED nnual Summary (1 Jul 99 - 30 Jun 02)		
4. TITLE AND SUBTITLE				(1 Jul 99 - 30 Jun 02) 5. FUNDING NUMBERS	
	Functional Analysis of TGF-a/b Regulated Protein			99-1-9172	
(TGFRP) in Breast Cancer Angiogenesis			, ,	· · · · · · · · · · · · · · · · · · ·	
(101K1) III DIEGBO CO	cci migrogenesis				
C AUTHORIC) -	·		*		
6. AUTHOR(S):					
Ryan M. Anderson					
			·		
7. DEDECOMING ODGANIZATION NAME	15/01 4 10 4 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)			8. PERFORMIN REPORT NU	IG ORGANIZATION	
Duke University Medi	cal Center		TIEL OIL ING	·	
Durham, North Caroli		·			
E-Mail:R.ANDERSON@CELLBIO.DUKE.ED			40 000000	1910 (1910)	
9. SPONSORING / MONITORING AGE	ENCY NAME(S) AND ADDRESS(ES	>)		ING / MONITORING REPORT NUMBER	
U.S. Army Medical Research and N	Materiel Command		AGENOT	JIII HUMULII	
Fort Detrick, Maryland 21702-501					
	·				
			•		
44 CURRIENTARY NOTES		***			
11. SUPPLEMENTARY NOTES				·	
			•		
12a. DISTRIBUTION / AVAILABILITY			• • • • • • • • • • • • • • • • • • • •	12b. DISTRIBUTION CODE	
12a. DISTRIBUTION / AVAILABILITY Approved for Public Rele		limited		12b. DISTRIBUTION CODE	
		limited		12b. DISTRIBUTION CODE	
		limited		12b. DISTRIBUTION CODE	
Approved for Public Rele	ease; Distribution Unl			12b. DISTRIBUTION CODE	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for	ease; Distribution Uni oct should contain no proprietary or confider or tumor growth, maintena	<u>dential information)</u> ance, and metastas		with no	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessity)	ease; Distribution Unict should contain no proprietary or confider tumor growth, maintend crotic, and incapable of	dential information) ance, and metastas intravasation and	l hematogeno	with no	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary transport. Therapeutic stra	ease; Distribution Unict should contain no proprietary or confider tumor growth, maintenance to tic, and incapable of attegies targeting vascula	dential information) ance, and metastas intravasation and arization promise	l hematogeno efficacy ag	with no us ainst all	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, need transport. Therapeutic stractypes of solid tumors, incl	ease; Distribution United should contain no proprietary or confider tumor growth, maintenserotic, and incapable of stegies targeting vasculating breast cancers. Provided the stage of the	dential information) ance, and metastas intravasation and arization promise reliminary studies	l hematogeno efficacy ag of the nov	with no us ainst all el secreted	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary transport. Therapeutic stractypes of solid tumors, incluprotein TGFRP suggest that vascularization, progression	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to an incapable of stegies targeting vasculations are to an important angion, and metastasis. The an importance on, and metastasis.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this projec	I hematogeno efficacy ag of the nov plicated in the is to elu	with no us ainst all el secreted tumor	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, need transport. Therapeutic stratypes of solid tumors, incluprotein TGFRP suggest that vascularization, progression role of TGFRP in carcinogenesis.	ease; Distribution United should contain no proprietary or confider tumor growth, maintense crotic, and incapable of stegies targeting vasculations breast cancers. Provided it is an important angion, and metastasis. The angles is by studying expressions.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this projects sion in normal dev	<pre>l hematogeno efficacy ag for the nov licated in for is to elu relopment,</pre>	with no us ainst all el secreted tumor cidate the	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, need transport. Therapeutic stratypes of solid tumors, incluprotein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to an angular tumor and incapable of tegies targeting vascular tumor breast cancers. Provided it is an important angular to an and metastasis. The angular tumors, and effects upon tumors, and effects upon the same and the s	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth	I hematogeno efficacy ag of the nov clicated in t is to elu relopment, delial cells	with no us ainst all el secreted tumor cidate the . We have	
Approved for Public Release 13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, need transport. Therapeutic stratypes of solid tumors, incluprotein TGFRP suggest that vascularization, progression role of TGFRP in carcinogenesis.	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to an incapable of stegies targeting vasculations an important angion, and metastasis. The an incapable of tumors, and effects upon tessed at the earliest stepsed sessed at the earliest stepsed sessed at the earliest stepsed at the earliest stepsed sessed	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev	Hematogeno efficacy age of the novelicated in the is to elu- relopment, delial cells relopment. He	with no us ainst all el secreted tumor cidate the . We have owever,	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary types of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemdothelial cells. Completi	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to an an incapable of stegies targeting vasculations an important angion, and metastasis. The an important and tumors, and effects upon tumors, and effects upon tessed at the earliest statem, we have found the state on of these studies will	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev IGFRP may act as a l provide an under	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary types of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemothelial cells. Completing TGFRP in normal and missing an inversal completing to the total cells.	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to the contain and incapable of stegies targeting vasculated breast cancers. Provided it is an important anging on, and metastasis. The angles is by studying expressing tumors, and effects upon the case of the earliest statem, we have found the case of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev IGFRP may act as a l provide an under	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary types of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemdothelial cells. Completi	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to the contain and incapable of stegies targeting vasculated breast cancers. Provided it is an important anging on, and metastasis. The angles is by studying expressing tumors, and effects upon the case of the earliest statem, we have found the case of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev IGFRP may act as a l provide an under	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary types of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemothelial cells. Completing TGFRP in normal and missing an inversal completing to the total cells.	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to the contain and incapable of stegies targeting vasculated breast cancers. Provided it is an important anging on, and metastasis. The angles is by studying expressing tumors, and effects upon the case of the earliest statem, we have found the case of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev IGFRP may act as a l provide an under	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary types of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemothelial cells. Completing TGFRP in normal and missing an inversal completing to the total cells.	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to the contain and incapable of stegies targeting vasculated breast cancers. Provided it is an important anging on, and metastasis. The angles is by studying expressing tumors, and effects upon the case of the earliest statem, we have found the case of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev IGFRP may act as a l provide an under	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary types of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemothelial cells. Completing TGFRP in normal and missing an inversal completing to the total cells.	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to the contain and incapable of stegies targeting vasculated breast cancers. Provided it is an important anging on, and metastasis. The angles is by studying expressing tumors, and effects upon the case of the earliest statem, we have found the case of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev IGFRP may act as a l provide an under	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, need transport. Therapeutic stratypes of solid tumors, incluprotein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemdothelial cells. Completi of TGFRP in normal and mist development of novel therap	ease; Distribution United should contain no proprietary or confider tumor growth, maintenance to the contain and incapable of stegies targeting vasculated breast cancers. Provided it is an important anging on, and metastasis. The angles is by studying expressing tumors, and effects upon the case of the earliest statem, we have found the case of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev IGFRP may act as a l provide an under	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstrated Angiogenesis is critical for vascular bed are small, need transport. Therapeutic strategyes of solid tumors, inclusively protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay system and the strategy of TGFRP in normal and missing development of novel theraped.	ease; Distribution United should contain no proprietary or confiner tumor growth, maintend crotic, and incapable of stegies targeting vasculated unding breast cancers. Profession, and metastasis. The alesis by studying expression, and effects upon tumors, and effects upon eased at the earliest statem, we have found the statem, we have found the statem of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev TGFRP may act as a l provide an under opment, and may co	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role the	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, need transport. Therapeutic stratypes of solid tumors, incluprotein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systemdothelial cells. Completi of TGFRP in normal and mist development of novel therap	ease; Distribution United should contain no proprietary or confiner tumor growth, maintend crotic, and incapable of stegies targeting vasculated unding breast cancers. Profession, and metastasis. The alesis by studying expression, and effects upon tumors, and effects upon eased at the earliest statem, we have found the statem, we have found the statem of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev TGFRP may act as a l provide an under opment, and may co	Hematogeno efficacy age of the novelicated in the ist of elument, delial cells relopment. He survival festanding of	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, need transport. Therapeutic stractypes of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogen inappropriate expression in found the TGFRP is not exprusing an in vitro assay systendothelial cells. Completi of TGFRP in normal and mist development of novel theraped. 14. SUBJECT TERMS breast cancer, angiogeness.	ease; Distribution United should contain no proprietary or confiner tumor growth, maintenance to the contained and incapable of itegies targeting vasculated unding breast cancers. Provided it is an important angion, and metastasis. The anesis by studying expressing tumors, and effects upon the case of the earliest statem, we have found the contained with the contained and the case of the	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev TGFRP may act as a l provide an under opment, and may co	hematogeno efficacy age of the novolicated in the ist to elurelopment, selial cells relopment. He survival frestanding of contribute to	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role the 15. NUMBER OF PAGES 10 16. PRICE CODE	
13. Abstract (Maximum 200 Words) (abstract Angiogenesis is critical for vascular bed are small, necessary types of solid tumors, include protein TGFRP suggest that vascularization, progression role of TGFRP in carcinogenesin in appropriate expression in found the TGFRP is not exprusing an in vitro assay system and the second and missing development of novel therapy. 14. SUBJECT TERMS breast cancer, angiogenesis	ease; Distribution United should contain no proprietary or confiner tumor growth, maintend crotic, and incapable of stegies targeting vasculated unding breast cancers. Profession, and metastasis. The alesis by studying expression, and effects upon tumors, and effects upon eased at the earliest statem, we have found the statem, we have found the statem of these studies will regulated vascular developments.	dential information) ance, and metastas intravasation and arization promise reliminary studies ogenic factor, imp aim of this project sion in normal dev on cultured endoth tages of mouse dev TGFRP may act as a l provide an under opment, and may co	hematogeno efficacy age of the novolicated in the ist to elurelopment, selial cells relopment. He survival frestanding of contribute to	with no us ainst all el secreted tumor cidate the . We have owever, actor for the role the	

Table of Contents

Cover	1
SF 298	2
Table of Contents	3
Introduction	4
Body	4
Key Research Accomplishments	6
Reportable Outcomes	6
Conclusions	6
References	6
Annendices	7

INTRODUCTION

The goal of these studies is to elucidate the roles of TGFRP (TGF α / β Regulated Protein) in breast cancer progression. We have been testing the hypothesis that TGFRP acts as an angiogenic factor in an autocrine and/or paracrine manner. Our findings thus far support this hypothesis. Elucidation of the angiogenic mechanism, of which TGFRP is likely a player, will reveal novel targets for breast cancer treatment which will in turn lead to new therapeutic strategies.

BODY

Task 1: Determine the expression pattern of *TGFRP*.

As previously reported, *TGFRP* is not expressed at detectable levels during early angiogenesis and vasculogenesis in mouse (embryonic day 6.5-11.5). To determine whether or not TGFRP has a role in later angiogenic/vasculogenic processes, we have examined expression in late gestation embryos (embryonic day 13.5-17.5) by *in situ* hybridization of paraffin sections. We have detected no specific signal above background levels with either antisense or sense control riboprobes. Several positive control riboprobes gave excellent results in these studies, giving us confidence that the protocol was working well. This suggests that TGFRP is either expressed at levels below the threshold of detection, or not all, during embryonic angiogenesis. Radioactive section *in situs* may prove to be more sensitive. Alternatively, TGFRP may play a role in homeostatic angiogenesis or vasculogenesis as might occur during wound healing or fracture repair (Gerber and Ferrara, 2000; Sherratt and Dallon, 2002). These studies are ongoing, although more effort has been channeled into furthering other tasks.

Task 2: Evaluate the role of TGFRP in angiogenesis

In vivo angiogenic studies of TGFRP as proposed originally have been postponed, as suitable resources are no longer available to us at Duke (see also previous report). We have therefore continued several lines of in vitro research. Our early results suggested that TGFRP may act on human microvascular endothelial cells (HMEC-1) in a non-autonomous manner. TGFRP acts as both a survival and morphogenetic factor (see earlier report). However, both human vascular endothelial cells (HUVEC) and HMEC-1 may be unsuitable for some angiogenic studies. HUVEC are derived from macrovascular source, while HMEC-1 cells have been immortalized

with large-T antigen, potentially altering their proliferative and survival responses (Pipas and Levine, 2001). We have therefore created a new cell line by immortalizing human microvascular endothelial cells (HMVEC) with the human telomerase catalytic subunit (hTERT). This method has been used successfully by others to immortalize cell lines (Salmon et al., 2000). Our cell line, dubbed IHMVEC, appears to retain characteristics of the primary line, and was used in subsequent proliferative studies.

We tested the mitogenic response of IHMVEC to TGFRP using a tritiated thymidine incorporation assay. These data demonstrate that TGFRP is not a mitogen for microvascular endothelial cells (Figure 1). These data do not refute the possibility that TGFRP is an angiogenic factor, but rather suggest that it may act in another manner. Two possibile functions include morphogenesis control and resistance to cellular stresses, such as hypoxia.

To test the first possibility, we performed long and short-term cell adhesion assays using TGFRP as an adhesive substrate. Although TGFRP does not promote adhesion after 90 minutes in culture (not shown), cell adhesion was significantly increased over BSA control after 24 hours (Figure 2). These data suggest that TGFRP may not act as a typical cell adhesion molecule, but rather through upregulation of other cell adhesion molecules or secretion of extracellular matrix components.

To test the second possibility that TGFRP promotes survival to cellular stresses, we mimicked hypoxia conditions through the addition of either CoCl₂ or DFO. Our data show that TGFRP-transfected MCF-7 cells survive in higher numbers that cells transfected with a control vector (Figure 3).

Task3: Identify and isolate the binding target of TGFRP

Isolation of the TGFRP receptor or binding target is necessary to fully characterize the signaling pathway activated by TGFRP. To that end we have successfully generated large quantities of high purity protein (as used above; Figure 4). Initial attempts at ¹²⁵I-labeling followed by binding assay were met with large background problems and other technical challenges, as described previously. To circumvent these problems we attempted to label the recombinant protein with biotin for the binding assay. Unfortunately, this method has proven not sensitive enough for binding detection. Thus, we have decided to proceed with the ¹²⁵I-labeling experiments, and are beginning to optimize the conditions.

While these experiments have been progressing we have also searched for downstream targets/effectors of TGFRP function. Intriguingly, we have found that

TGFRP may upregulate the expression of two receptor tyrosine kinases involved in angiogenesis: Flk-1, a receptor for VEGF, and Tie-2, a receptor for Ang-1 (Figure 5). These receptors may have roles both in de novo vessel formation as well as remodeling of existing vascular networks reviewed in (Liu et al., 2000). These data suggest that TGFRP may act in part by increasing the signal transduction through known angiogenic pathways.

KEY RESEARCH ACCOMPLISHMENTS

- 1. Determination that TGFRP is not expressed embryonically in the mouse.
- 2. Further support that TGFRP does not act directly as a mitogen for endothelial cells.
- 3. determination that TGFRP may increase cell adhesion, possibly by an indirect mechanism.
- 4. Determination that TGFRP may protect cells from hypoxia stress.
- 5. Determination that TGFRP may upregulate the expression of receptor tyrosine kinases important in known pathways of angiogenesis.

REPORTABLE OUTCOMES

All category of activities are in progress.

CONCLUSIONS

The studies summarized by this report provide support for the hypothesis that TGFRP promotes the growth and survival of breast tumors by promoting blood vessel morphogenesis, promoting other angiogenic signaling pathways, and protecting against the stresses of nutrient and oxygen depletion. While TGFRP has not been detected during mouse embryonic development, its expression may be tightly reguated at a level below the threshold of sensitivity of our analysis. Aside from this, it remains that TGFRP may be important for other homeostatic mechanisms in newborn and adult animals.

REFERENCES

- **Gerber, H. P. and Ferrara, N.** (2000). Angiogenesis and bone growth. *Trends Cardiovasc Med* **10**, 223-228.
- Liu, W., Ahmad, S. A., Reinmuth, N., Shaheen, R. M., Jung, Y. D., Fan, F. and Ellis, L. M. (2000). Endothelial cell survival and apoptosis in the tumor vasculature. *Apoptosis* **5**, 323-328.

- **Pipas, J. M. and Levine, A. J.** (2001). Role of T antigen interactions with p53 in tumorigenesis. *Semin Cancer Biol* **11**, 23-30.
- Salmon, P., Oberholzer, J., Occhiodoro, T., Morel, P., Lou, J. and Trono, D. (2000). Reversible immortalization of human primary cells by lentivector- mediated transfer of specific genes. *Mol Ther* 2, 404-414.
- **Sherratt, J. A. and Dallon, J. C.** (2002). Theoretical models of wound healing: past successes and future challenges. *C R Biol* **325**, 557-564.

APPENDIX

Four figures which are referenced in the body of this report.

APPENDIX

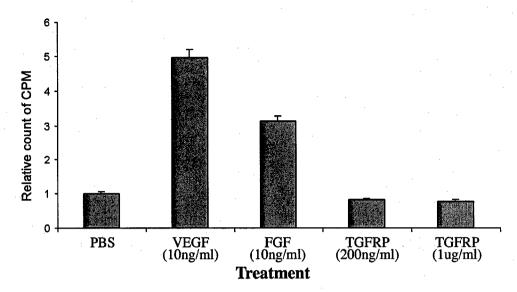


Figure 1. Tritiated thymidine incorporation assay. Serum-starved IHMVEC were treated with PBS, VEGF, FGF, or TPF at the indicated concentrations. Following 10 hours of culture, labeled thymidine was added and incubated for 4 additional hours. Cells were lysed, and labeled thymidine was quantitated by a scintillation counter.

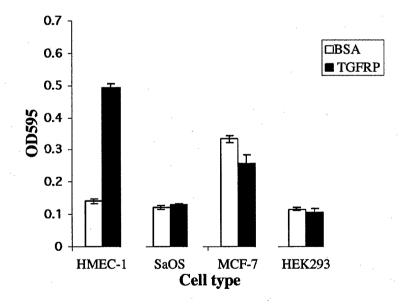


Figure 2. HMEC-1 long-term adhesion assay on TGFRP substrate. 96-well plates were coated with 10ug/ml TGFRP overnight at 4C, then blocked with BSA. HMEC-1 cells were plated at a density of 10⁴ cells/well, and incubated for 24 hours. Wells were then washed with PBS and attached cells were stained with crystal violet. The remaining dye was quantitated using a plate reader.

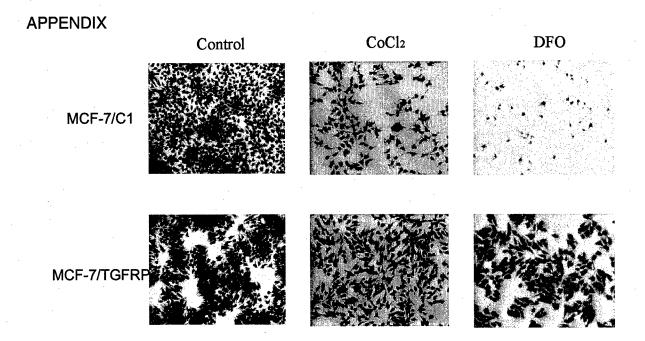


Figure 3. TGFRP-transfected MCF-7 cells are protected from hypoxia stress. Stable transfectants (MCF-7/TGFRP) and control cells (MCF-7/C1) were treated with either CoCl₂ or DFO for three days in serum-free conditions. Surviving cells were stained with DiffQuick.

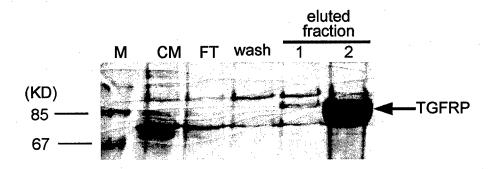


Figure 4. Purification of recombinant His(6)-tagged TGFRP from baculovirus. Conditioned media was loaded onto a nickel column (Qiagen), washed with 10mM imidazole, and eluted with 300mM imidazole

APPENDIX

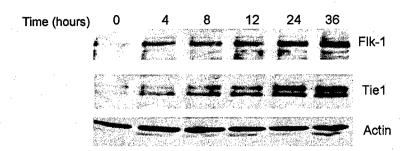


Figure 5. Induction of angiogenic receptors in IHMVEC by TGFRP. IHVEC were treated with TGFRP at a concentration of 200ng/ml. Cells were lysed, equally loaded into an SDS-PAGE gel, and transferred to a nitrocellulose membrane. Protein was detected with α -Flk-1, α -Tie-1, or α -actin antibodies as indicated.